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14. Adamczewski, M., Köhler, G. & Lamers, M. C. *Eur. J. Immun.* **21**, 617–626 (1991).
15. Liu, Y.-J., Johnson, G. D., Gordon, J. & MacLennan, I. C. M. *Immun. Today* **13**, 17–21 (1992).
16. Maeda, K. *et al. J. Immun.* **148**, 2340–2347 (1992).
17. Heyman, B., Tianmin, L. & Gustavsson, S. *Eur. J. Immun.* **23**, 1739–1742 (1993).
18. Lebrun, P. & Spiegelberg, H. L. *J. Immun.* **139**, 1459–1465 (1987).
19. Kopf, M. *et al. Nature* **362**, 245–247 (1993).
20. Keegan, A. D., Snapper, C. M., Van Dusen, R., Paul, W. E. & Conrad, D. H. *J. Immun.* **142**, 3868–3874 (1989).
21. Luo, H., Hofstetter, H., Banchereau, J. & Delespesse, G. *J. Immun.* **146**, 2122–2129 (1991).
22. Shen, E., Macy, E., Kimata, H., Gilly, M. & Saxon, A. *J. Immun.* **142**, 481–489 (1989).
23. Saxon, A., Kurbe-Leamer, M., Behle, K., Max, E. E. & Zheng, K. *J. Immun.* **147**, 4000–4006 (1991).
24. Street, N. E. *et al. J. Immun.* **144**, 1629–1639 (1990).
25. Campbell, K. A., Lees, A., Finkelman, F. D. & Conrad, D. H. *Eur. J. Immun.* **22**, 2107–2112 (1992).
26. Sinclair, N. R. StC. *Immun. Today* **4**, 35–36 (1983).
27. Nitschke, L., Kopf, M. & Lamers, M. C. *BioTechniques* **14**, 914–916 (1993).
28. Ayane, M. P., Nielsen, P. & Köhler, G. *Nucleic Acids Res.* **17**, 6722 (1990).
29. Rao, M., Lee, W. T. & Conrad, D. H. *J. Immun.* **138**, 1845–1851 (1987).

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A mammalian protein targeted by G1-arresting rapamycin–receptor complex

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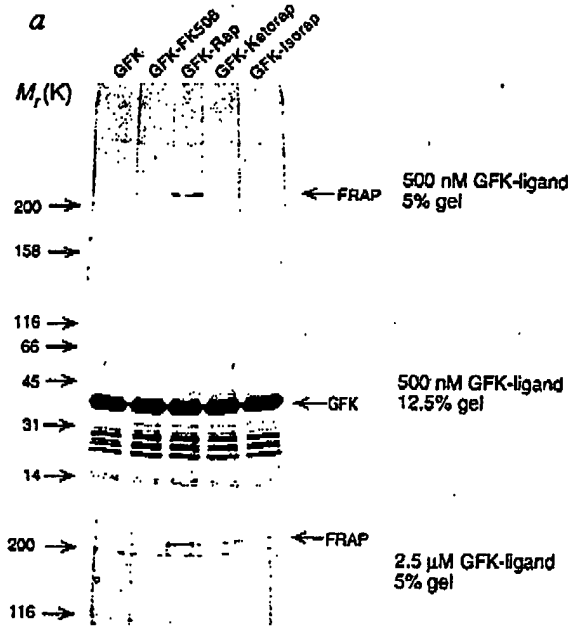
THE structurally related natural products rapamycin and FK506 bind to the same intracellular receptor, FKBP12, yet the resulting complexes interfere with distinct signalling pathways[1,2]. FKBP12–rapamycin inhibits progression through the G1 phase of the cell cycle in osteosarcoma[3], liver[4,5] and T cells[6,7] as well as in yeast[8], and interferes with mitogenic signalling pathways that are involved in G1 progression[9,10], namely with activation of the protein $p70^{S6k}$ (refs 5, 11–13) and cyclin-dependent kinases[3,14–16]. Here we isolate a mammalian FKBP–rapamycin-associated protein (FRAP) whose binding to structural variants of rapamycin complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression. Peptide sequences from purified bovine FRAP were used to isolate a human cDNA clone that is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products from *Saccharomyces cerevisiae*[8,17,18]. Although it has not been previously demonstrated that either of the *DRR/TOR* gene products can bind the FKBP–rapamycin complex directly[17,19], these yeast genes have been genetically linked to a rapamycin-sensitive pathway and are thought to encode lipid kinases[17–20].



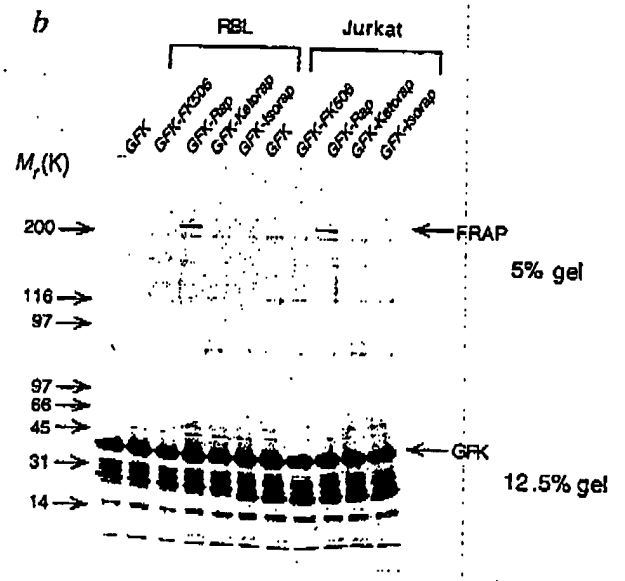
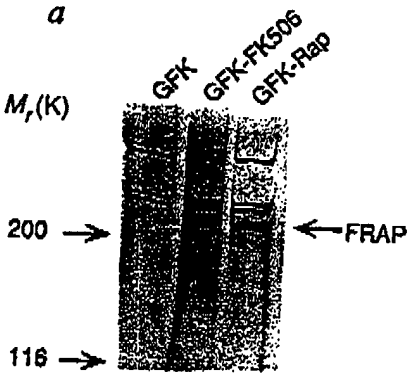


FIG. 1 Identification of FRAP protein in three mammalian cell lines. *a*, GFK alone or individual GFK–ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μM and the mixtures incubated for 10 min at 4 °C. Fusion protein complexes were recovered by glutathione-affinity chromatography, and the proteins detected by silver staining after 5% SDS–PAGE. Because of compression, FRAP is not resolved by 12.5% SDS–PAGE, so both 5% and 12.5% gels are shown. The amount of FRAP that was retained by affinity chromatography saturated at concentrations of GFK–Rap greater than 500 nM in these experiments and in others using concentrations of GFK–Rap ranging from 100 nM to 5 μM (data not shown). *b*, GFK alone or individual GFK–ligand complexes were added to a final concentration of 500 nM to lysates prepared from either 2×10^8 Jurkat T lymphocytes or 10^8 rat basophilic leukaemia (RBL) cells per condition. Lysates were treated as in *a*. FKBP12, but not FKBP13 or FKBP25 (ref. 23) is able to mediate the actions of rapamycin in *S. cerevisiae*. In addition, we found that YFK188 (ref. 24), an FKBP12 null strain, could be complemented with GFK (P. K. Martin, B. Gladstone, G. Weiss, D. T. Hung, S.L.S., in preparation). Thus the GST appendage of the fusion protein does not preclude binding of the biologically relevant target to the GFK–rapamycin complex in yeast.

METHODS. MG-63, Jurkat and RBL cells were grown in media containing 10% FBS and lysed at 4 °C in PINT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A and 2 mM DTT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000*g*, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK prebound to stoichiometric quantities of FK506, keto- iso- or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250-μl glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.

EXHIBIT D



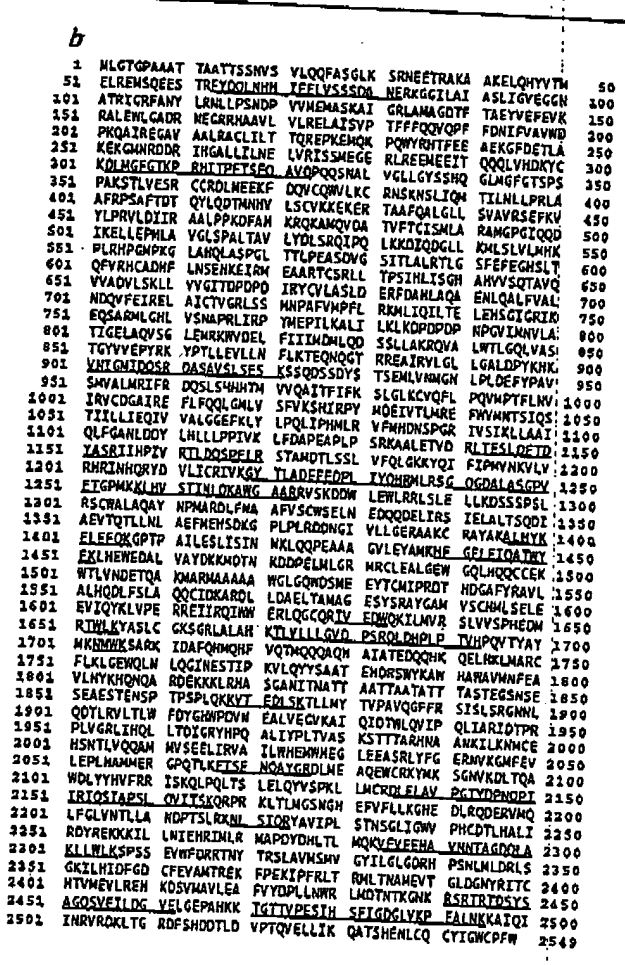


FIG. 2 Purification of FRAP from bovine brain and cDNA cloning of human FRAP. *a*, Fivefold-enriched bovine FRAP (S-column eluate; see below) was conditioned with [100 nM], glutathione-S-transferase-FKBP12 fusion protein (GFK), GFK-FK506 or GFK–Rap. Complexes with fusion proteins were recovered by glutathione-affinity chromatography and detected as described in Fig 1 legend. We also found FRAP in bovine liver and thymus. *b*, Predicted translational product of the human FRAP cDNA clone. Bovine FRAP peptide sequences aligned to human FRAP are indicated by underlined segments. In the reading frame shown translational stop codons were not encountered upstream of the initiating methionine. *c*, Northern blot analysis of human tissue, Jurkat T cell and MG-63 cell poly (A)$^+$ RNA. The Jurkat/MG-63 and multiple tissue Northern blots (Clontech) were hybridized with ^{32}P-labelled probes derived from the 182 bp PCR fragment and the 5.5 kb clone (text), respectively. Hybridization to human β-actin probe is shown as an internal control for loading.

METHODS. Bovine FRAP was purified by grinding 900 g of bovine brain in blender with 1 litre of PIP (0.3% Triton X-100, 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μM Na_3VO_4, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 mM benzamidine and 2 mM DTT). The homogenate was centrifuged at 25,000*g* and the supernatant (20 g total protein) was loaded onto a 1 litre S-Sepharose (Pharmacia) column. The column was then washed with PIP and eluted with PINP (PIP with 1M NaCl). GFK–rapamycin was added to the pooled eluate to a final concentration of 100 nM and recovered by glutathione-affinity chromatography. FRAP was resolved by SDS–PAGE and transferred to PVDF. Following digestion with trypsin or endoproteinase Lys-C (Boehringer Mannheim) bFRAP peptides were microsequenced[23]. The Jurkat T cell cDNA library (Stratagene) was constructed through random and oligo dT priming of cytoplasmic oligo dT purified RNA (ref. 25). cDNA screening, Jurkat and MG-63 RNA isolation and northern blotting and were performed by procedures similar to those previously described[25]. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ^{32}P-dCTP in the course of reamplification by PCR. The sequences were analysed using BLAST (ref. 26) and the University of Wisconsin GCG (ref. 27) software. The human FRAP cDNA sequence has been submitted to Genbank.

We used two structural variants of rapamycin, 16-keto-rapamycin (S. D. Meyer and S.L.S., manuscript in preparation) and 25,26-iso-rapamycin[21], to identify any biologically relevant targets of the FKBP–rapamycin complex. Both variants bind tightly to human FKBP12, as shown by their ability to inhibit rotamase activity of the recombinant protein (K_i values were 0.2 nM for rapamycin[6], 2 nM for keto-rapamycin, and 0.1 nM for iso-rapamycin). But the variants are about two orders of magnitude less potent than rapamycin in preventing the progression through G1 of MG-63 human osteosarcoma cells. The values of IC_{50} (half-maximal inhibitory concentration) estimated from dose–response curves are 0.1 nM, 7.5 nM and 50 nM for rapamycin, keto- and iso-rapamycin, respectively. Thus the complexes of iso- and keto-rapamycin with FKBP12 should bind to the FKBP12–rapamycin target less effectively than FKBP12–rapamycin itself.

A fusion protein of glutathione-*S*-transferase with FKBP12 (GFK) was used to identify candidates for the biologically relevant targets of FKBP12–rapamycin. MG-63 cells were lysed by detergent and complexes of GFK–rapamycin, GFK–FK506 or GFK alone were added individually to clarified lysate at a final concentration of 500 nM or 2.5 μM (Fig. 1*a*). A protein of approximate relative molecular mass 220,000 (M_r ~220K) was detected in the GFK–rapamycin sample by SDS–PAGE and silver staining (Fig. 1*a*, lane 3). This FKBP–rapamycin-associated protein (FRAP) was not retained with GFK–FK506 or GFK alone (Fig. 1*a*, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1*a*) or by a similar